F(ab)′$_2$-mediated neutralization of C3a and C5a anaphylatoxins: a novel effector function of immunoglobulins

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Published online 3 March 2003; doi:10.1038/nm836

High-dose intravenous immunoglobulin (IVIG) prevents immune damage by scavenging complement fragments C3b and C4b. We tested the hypothesis that exogenous immunoglobulin molecules also bind anaphylatoxins C3a and C5a, thereby neutralizing their pro-inflammatory effects. Single-cell calcium measurements in HMC-1 human mast cells showed that a rise in intracellular calcium caused by C3a and C5a was inhibited in a concentration-dependent manner by IVIG, F(ab)′$_2$-IVIG and irrelevant human monoclonal antibody. C3a- and C5a-induced thromboxane (TXB_2) generation and histamine release from HMC-1 cells and whole-blood basophils were also suppressed by exogenous immunoglobulins. In a mouse model of asthma, immunoglobulin treatment reduced cellular migration to the lung. Lethal C5a-mediated circulatory collapse in pigs was prevented by pretreatment with F(ab)′$_2$-IVIG. Molecular modeling, surface plasmon resonance (SPR) and western blot analyses suggested a physical association between anaphylatoxins and the constant region of F(ab)′$_2$. This binding could interfere with the role of C3a and C5a in inflammation.

IVIG has been reported to be therapeutic in a variety of (auto)immune diseases[1]. However, the mechanisms of action of IVIG are still under study. One hypothesis, based on *in vivo* models of complement-mediated diseases, *in vitro* assays and patient studies[2–7], is that infused immunoglobulin molecules interact with complement fragments C3b and C4b, which are effectors of the complement cascade on the cell surface. This scavenging of C3b and C4b before they reach target tissues prevents the destruction of target cells[8].

C3a and C5a, smaller proteolytic fragments of complement components 3 and 5, are known as anaphylatoxins. They are released from their respective native components after activation of the complement cascade. These molecules induce potent biological responses at very low concentrations and are involved in inflammatory and anaphylactic reactions. Their receptors have been identified on circulating leukocytes, mast cells, macrophages, hepatocytes, lung epithelial cells, endothelial cells, astrocytes and brain microglial cells[9]. Activation of these cells by anaphylatoxins results in the release of inflammatory mediators[10], which in turn alter vascular permeability, induce smooth muscle contraction and cause inflammatory cell migration. In addition, anaphylatoxins amplify the inflammatory response by inducing the synthesis of pro-inflammatory cytokines[11]. This anaphylatoxin-triggered cascade of events contributes to the pathogenesis of a number of diseases and conditions, including but not restricted to hypersensitivity reactions, myocardial ischemia–reperfusion injury, endotoxic shock, multiple organ failure, respiratory distress syndrome and neurodegenerative diseases[9].

Neutralization of the effects of anaphylatoxins is therefore of substantial clinical significance. It has been reported that C3a binds to IgG molecules[12–14], although the consequences on its effector functions have not been studied. Nothing is known about the ability of C5a to associate with IgG.

The working hypothesis of this study was that therapeutic doses of exogenous immunoglobulins would functionally inactivate C3a and C5a. We show that C3a and C5a seem to bind to immunoglobulin molecules through the constant region of

F(ab)′$_2$ fragments. This interaction inhibits anaphylatoxin-induced pro-inflammatory events *in vitro* and *in vivo*.

Single-cell calcium imaging studies in HMC-1 cells

To examine the effect of IgG on the ability of C3a and C5a to mobilize calcium from intracellular pools we used HMC-1 cells, which possess receptors for both C3a and C5a[15]. Initially, we characterized the pattern of the C3a- and C5a-induced rise in intracellular calcium concentration ($[Ca^{2+}]_i$) in single cells. Optimal $[Ca^{2+}]_i$ response in HMC-1 cells was obtained by stimulating them with 10 nM C5a, 56 nM C3a or both, alone or sequentially (Fig. 1*a*). These data indicate that C3a and C5a activate the calcium mobilization pathway in HMC-1 cells, confirming previous findings in a bulk cell assay[15].

We then pre-incubated C3a and C5a with two different IVIG preparations, one containing whole IgG molecules (Venimmun N; referred to here as IVIG) and the other consisting predominantly of the F(ab)′$_2$ portion of IgG (GammaVenin; referred to here as F(ab)′$_2$-IVIG). Both preparations at 25 mg/ml blocked $[Ca^{2+}]_i$ responses induced by optimum concentrations of C3a and C5a (Fig. 1*b* and *c*). Higher concentrations of both anaphylatoxins (up to 112 nM), similar to amounts measured in pathological conditions[16], were likewise suppressed by these doses (data not shown). Human monoclonal antibody HB8501 against tetanus toxoid, at 25 mg/ml, abrogated C3a- and C5a-induced $[Ca^{2+}]_i$ responses (Fig. 1*b* and *c*). C3a- and C5a-induced calcium signaling was suppressed as a function of increasing concentrations of both IVIG preparations and monoclonal antibody HB8501; the half-maximal effective concentrations were in the range of 10–15 mg/ml (Fig. 1*d* and *e*).

We performed controls to determine the specificity of the inhibitory effect of immunoglobulins, as well as the identity of the immunoglobulin fragment responsible for suppression. Fc fragments at 25 mg/ml did not suppress $[Ca^{2+}]_i$ responses after pre-incubation with anaphylatoxins (Fig. 1*d* and *e*), nor did albumin at 25 mg/ml or the vehicle for both IVIG preparations (glycine at 26.2 mg/ml) (Table 1). Transforming growth factor (TGF)-β2 is a cytokine that can exhibit immunosuppressive effects and is known to be present in some IVIG preparations, including Venimmun[17]. Recombinant TGF-β2 at 150 ng/ml did not inhibit the ability of anaphylatoxins to induce calcium responses in HMC-1 cells (Table 1). Platelet-activating factor (PAF) causes an increase in cytosolic calcium in HMC-1 cells[15]. This response was not altered by pre-incubation of PAF with IVIG at 25 mg/ml (Table 1). Similarly, serum-free culture medium failed to inhibit C3a- and C5a-triggered calcium flux (data not shown).

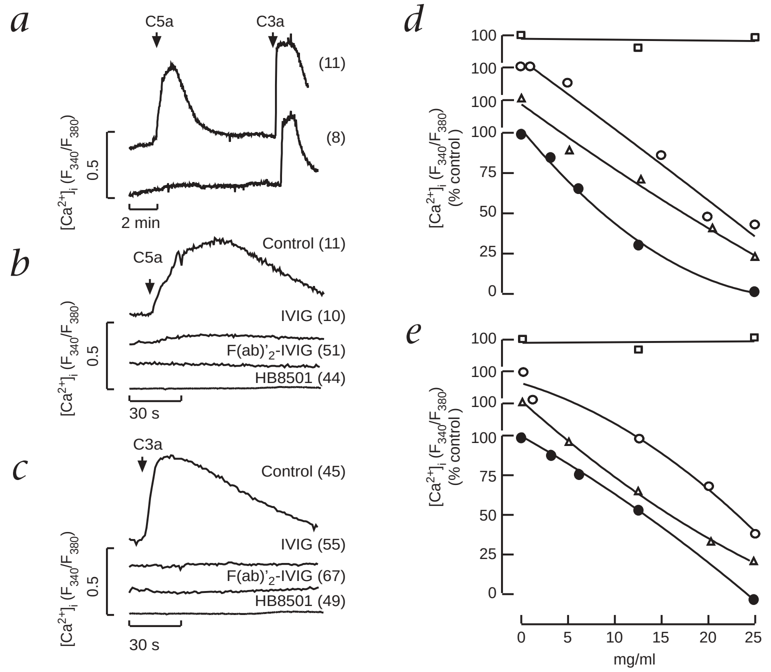

Fig. 1 C3a- and C5a-induced $[Ca^{2+}]_i$ responses in HMC-1 cells are inhibited by immunoglobulins. ***a***, $[Ca^{2+}]_i$ response in HMC-1 cells. Cells were stimulated with 10 nM C5a followed by 56 nM C3a (upper tracing) or 56 nM C3a alone (lower tracing). Shown are means from individual cell recordings (number of individual cells is indicated in parentheses), representative of 13 similar experiments. ***b*** and ***c***, Inhibition of C5a- and C3a-induced $[Ca^{2+}]_i$ responses by IVIG, F(ab)′$_2$-IVIG and the irrelevant human monoclonal antibody HB8501. The response of HMC-1 cells to C5a (*b*) and C3a (*c*) at the above concentrations was recorded (upper tracings); or anaphylatoxins were incubated with IVIG, F(ab)′$_2$-IVIG and human monoclonal antibody before addition to cells (lower 3 tracings). Shown are means of individual cell recordings. Numbers in brackets indicate individual cells. Representative of 3 similar experiments. ***d*** and ***e***, Dose-dependent inhibition of C3a (*d*)- and C5a (*e*)-induced $[Ca^{2+}]_i$ responses in HMC-1 cells by immunoglobulins. Data points are means derived from individual cell recordings (*n*= 35–96 or 43–69 per point for *d* and *e*, respectively). Tested were IVIG (○), F(ab)′$_2$-IVIG (●), human monoclonal antibody (△) or Fc IgG fragment (□). Standard errors were within 10% of the means. Inhibition of $[Ca^{2+}]_i$ responses is expressed as percent control (percentage of $[Ca^{2+}]_i$ in HMC-1 cells stimulated with respective anaphylatoxin alone). Representative of 3 similar experiments.

Screening for specific anaphylatoxin inhibitors

The carboxypeptidases N and R are enzymes that inactivate anaphylatoxins by cleaving their C-terminal arginine residues[18]. To see if carboxypeptidases might explain the observed neutralization of anaphylatoxins, we used a sensitive colorimetric assay to screen our IVIG and F(ab)′$_2$-IVIG preparations. We detected carboxypeptidases in normal human plasma but not in IVIG preparations (data not shown).

Inhibition of mediator release from HMC-1 cells

Histamine is stored in cytoplasmic granules and is actively secreted when mast cells are challenged with anaphylatoxins[10]. Addition of C3a or C5a to HMC-1 cell suspensions induced a 32% and 37% release of total histamine, respectively, whereas compound 48/80, a mast cell stimulant, induced an 85% release (Fig. 2*a*). Pre-incubation of C3a or C5a with IVIG at 15 mg/ml inhibited anaphylatoxin-triggered histamine release by 85% and 80%, respectively; F(ab)′$_2$-IVIG at the same dose reduced the release to background values (Fig. 2*b*). Pre-incubation of C3a and C5a with albumin (15 mg/ml) and vehicle did not affect mediator release by HMC-1 cells (Fig. 2*b*). The histamine-releasing ability of compound 48/80 was not affected by pre-incubation with IVIG at 15 mg/ml (223 ± 14 ng of histamine released per 10^5 cells with 48/80 alone; 237 ± 16 ng per 10^5 cells with 48/80 pre-incubated with IVIG).

Thromboxanes are oxidation products of arachidonic acid with pro-inflammatory properties[19]. We measured TXB_2 in supernatants of HMC-1 cells. After addition of C3a (56 nM) or C5a (10 nM), there was a release of 90.5 ± 11.4 pg/well and 82.6 ±

Table 1 Effect of control reagents on anaphylatoxin-induced calcium mobilization from HMC-1 cells.

		Intracellular calcium (F_{340}/F_{380})	
Treatment	Dose (mg/ml)	C3a	C5a
Albumin	0.0	.410 (45)[a]	.297 (49)
	25.0	.450 (51)	.287 (54)
Glycine	0.0	.397 (55)	.312 (57)
	26.2	.418 (39)	.336 (37)
TGF-β	0.0	.653 (47)	.376 (55)
	150.0 ng/ml	.642 (51)	.391 (62)
		PAF	
IVIG	0.0	.697 (58)	
	25.0	.689 (61)	

[a]Numbers in parentheses indicate total number of HMC-1 cells used per experiment; each is representative of 3 similar experiments.

10.2 pg/well TXB_2, respectively. Phorbol myristate acetate (PMA) caused the release of 121.4 ± 14.1 pg/well TXB_2 (Fig. 2*c*). Pre-incubation of C3a or C5a with F(ab)′$_2$ at 15 mg/ml caused almost complete inhibition of TXB_2 release. Similarly, pretreatment with 15 mg/ml of IVIG caused substantial inhibition of TXB_2 release. Incubation of these anaphylatoxins with albumin or vehicle alone did not inhibit their capacity to cause TXB_2 release (Fig. 2*d*).

To examine mediator release under physiologic conditions, samples of heparinized whole blood obtained from a healthy human donor were stimulated with optimal doses of C3a and C5a to obtain baseline histamine-release values for basophils (Fig. 2*e*). Blood samples were then supplemented with increasing doses of IVIG, followed by C3a or C5a. Inhibition of histamine release was directly proportional to IVIG dose, for both C3a and C5a (Fig. 2*f*).

Suppression of cellular migration in mouse asthma model

The hallmarks of an asthma-like condition, induced in mice by systemic sensitization and airway challenge with ovalbumin (OA), include pulmonary inflammation characterized by an increase in the number of eosinophils in bronchoalveolar lavage (BAL) fluid, and the presence of perivascular and peribronchial cellular infiltrates in lung tissue sections[20]. Our preliminary kinetic study showed peak BAL eosinophilia and cellular infiltration of lung tissues 72 h after challenge (data not shown). We therefore conducted all subsequent experiments at this time point. Analysis of BAL showed that IVIG, F(ab)′$_2$-IVIG and human monoclonal antibody HB8633 against a cancer antigen, administered at 2 mg per g body weight before OA challenge, reduced the number of eosinophils in the bronchoalveolar compartment in a concentration-responsive manner (Fig. 3*a*–*d*). IVIG treatment administered two days after challenge inhibited BAL eosinophilia to almost the same degree as IVIG pretreatment (Fig. 3*a*). Treatment with human serum albumin, glycine or cell culture medium had no effect on the number of eosinophils in the BAL fluid of Balb/c mice (data not shown); the BAL eosinophil number in those mice did not differ substantially from that of the PBS-treated groups (Fig. 3*a*). B6/129 F_1/J mice with *C3* gene deletion (*C3*$^{-/-}$), compared with their wild-type littermates and with Balb/c mice, showed a lower number of eosinophils in BAL fluid after ovalbumin sensitization and challenge. This reduction was quantitatively similar to that observed in Balb/c mice subjected to immunoglobulin treatment (Fig. 3*a*).

Analysis of the corresponding lung tissue sections showed extensive perivascular and peribronchial infiltrates in PBS-treated Balb/c and wild-type B6/129 F_1/J mice (Fig. 3*e* and *h*). IVIG and F(ab)′$_2$-IVIG treatment led to a reduction in the number and extent of infiltrates in the lung parenchyma (Fig. 3*f* and *g*), indistinguishable from what is observed in the lungs of *C3*$^{-/-}$ mice (Fig. 3*i*). Histological changes observed in the lungs of mono-

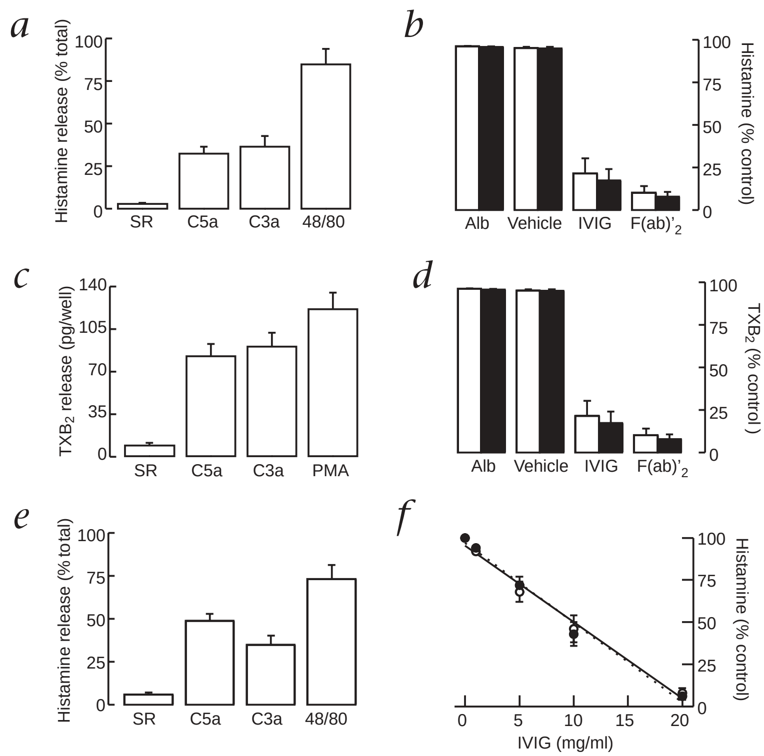

Fig. 2 Inhibition of C3a- and C5a-induced release of histamine and thromboxane from HMC-1 cells and whole-blood basophils. ***a***, Release of histamine after stimulation of HMC-1 cells with 56 nM C3a, 10 nM C5a or compound 48/80, a positive control. SR, spontaneous release of histamine from resting cells. Values are percentage of total histamine content. ***b***, Inhibition of histamine release by IVIG, F(ab)′$_2$-IVIG and control reagents. C3a (■) and C5a (□) were pre-incubated at the above concentrations with IVIG, F(ab)′$_2$-IVIG preparations, vehicle for IVIG and albumin (Alb). Inhibition of histamine release is expressed as percent control, obtained by incubating HMC-1 cells with anaphylatoxins alone. ***c***, TXB_2 in supernatants of HMC-1 cells with buffer alone (SR) or after 6-h stimulation with C5a, C3a and PMA. ***d***, Inhibition of TXB_2 release by pre-incubation of C3a (■) and C5a (□) with IVIG, F(ab)′$_2$-IVIG, vehicle for IVIG and albumin, expressed as percent control (thromboxane release caused by the anaphylatoxins alone). ***e***, Release of histamine from whole-blood basophils after stimulation with C3a and C5a at the above concentrations. SR, spontaneous release from unstimulated whole-blood samples. Values are percentage of total histamine content. ***f***, Dose-dependence of inhibition of C3a (●)- and C5a (○)-triggered histamine release from whole-blood basophils by IVIG. Results expressed as percentage of control, as described for (*b*). Data are mean ± s.e.m. of triplicate measurements, representative of 3 similar experiments.

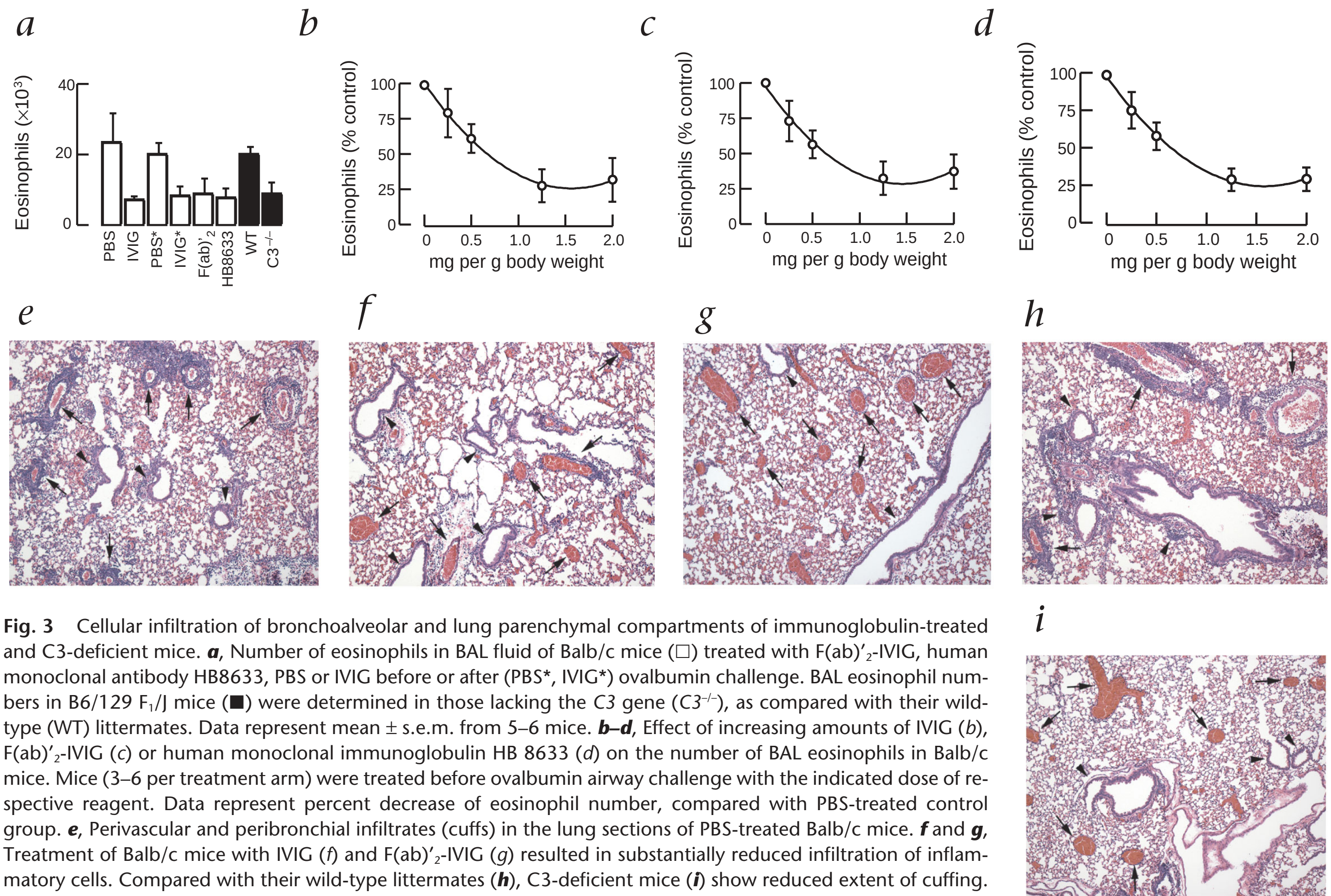

Fig. 3 Cellular infiltration of bronchoalveolar and lung parenchymal compartments of immunoglobulin-treated and C3-deficient mice. ***a***, Number of eosinophils in BAL fluid of Balb/c mice (□) treated with F(ab)′$_2$-IVIG, human monoclonal antibody HB8633, PBS or IVIG before or after (PBS*, IVIG*) ovalbumin challenge. BAL eosinophil numbers in B6/129 F$_1$/J mice (■) were determined in those lacking the *C3* gene (*C3*$^{-/-}$), as compared with their wild-type (WT) littermates. Data represent mean ± s.e.m. from 5–6 mice. ***b–d***, Effect of increasing amounts of IVIG (*b*), F(ab)′$_2$-IVIG (*c*) or human monoclonal immunoglobulin HB 8633 (*d*) on the number of BAL eosinophils in Balb/c mice. Mice (3–6 per treatment arm) were treated before ovalbumin airway challenge with the indicated dose of respective reagent. Data represent percent decrease of eosinophil number, compared with PBS-treated control group. ***e***, Perivascular and peribronchial infiltrates (cuffs) in the lung sections of PBS-treated Balb/c mice. ***f*** and ***g***, Treatment of Balb/c mice with IVIG (*f*) and F(ab)′$_2$-IVIG (*g*) resulted in substantially reduced infiltration of inflammatory cells. Compared with their wild-type littermates (***h***), C3-deficient mice (***i***) show reduced extent of cuffing. *e–i*, H&E staining. Arrows, blood vessels; arrowheads, bronchi. Magnification ×25.

clonal antibody–treated mice (data not shown) were identical to those of IVIG-treated animals (Fig. 3*f* and *g*). The number of perivascular cuffs was much lower in F(ab)′$_2$-IVIG (41 ± 15.7), IVIG (36.6 ± 10.1) and monoclonal antibody–treated (39.2 ± 9.5) mice, relative to PBS and medium-treated Balb/c controls (77.8 ± 14 and 81.0 ± 11, respectively). Similarly, perivascular infiltrates were much more abundant in wild-type B6/129 F$_1$/J mice (81 ± 18) than in their *C3*$^{-/-}$ littermates (29.2 ± 9.9).

Inhibition of a lethal C5a-dependent reaction in pigs

To provide additional evidence of *in vivo* anaphylatoxin neutralization by intravenous immunoglobulins, we used a porcine model of anaphylatoxin-induced cardiopulmonary distress. This model, using closed-chest-instrumented pigs, is highly sensitive, reproducible and relevant to human pathology, especially that of complement-mediated anaphylactic reactions[21]. Human C5a at 40 ng/kg was administered intravenously to pigs (n = 2). Profound hemodynamic changes, including maximal rise and subsequent complete loss of pulmonary arterial pressure (PAP) with associated steep decline and loss of systemic arterial pressure, were observed within several minutes (Fig. 4*a*). Another group of two animals was pretreated by infusion of F(ab)′$_2$-IVIG at 300 mg/kg. The lethal circulatory collapse caused by 40 ng/kg of C5a was prevented by such treatment, even after two subsequent C5a challenges that represented 4 and 12 times the lethal dose for unprotected pigs (Fig. 4*b*).

Demonstration of anaphylatoxin/immunoglobulin binding

We used western blot analysis to study the formation of anaphylatoxin-immunoglobulin complexes and their role in inhibition of C3a and C5a. Pre-incubation of C3a with purified F(ab)′$_2$ fragments caused a shift in the molecular weight of C3a, as detected by C3a-specific monoclonal antibody, from 9 kD (C3a alone; Fig. 5*a*, lane 1) to ~120 kD [C3a pre-incubated with

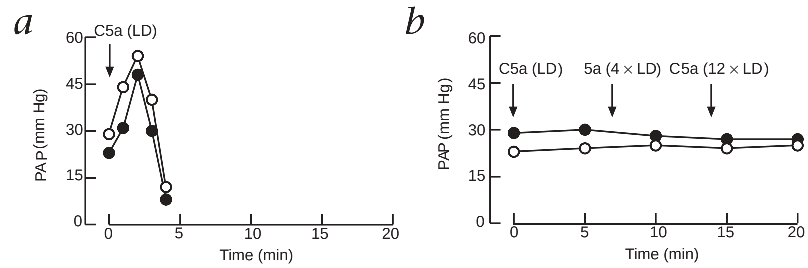

Fig. 4 Inhibition of C5a-induced lethal circulatory changes in pigs. ***a***, Pigs were treated with 40 ng/kg of human C5a and PAP was recorded. ***b***, Pigs were pretreated with F(ab)′$_2$-IVIG 30 min before injection of the same dose of C5a that was lethal for control animals. PAP was monitored throughout this and the period of 2 additional injections of C5a. *a* and *b*, ○, ● each represent 1 animal (total n = 4). LD, lethal dose.

F(ab)′$_2$], indicating physical association of C3a with F(ab)′$_2$ (Fig. 5*a*, lane 4). Pre-incubation of anaphylatoxins with the Fc fragment of the IgG molecule or with albumin (Fig. 5*a*, lanes 2 and 3) had no effect on their electrophoretic mobility, consistent with specific anaphylatoxin-F(ab)′$_2$ binding. Similar results (data not shown) were obtained for C5a using C5a-specific monoclonal antibody; the antigen-antibody shift was from 11 kD (C5a alone) to ~120 kD (C5a pre-incubated with monoclonal antibody). Next, C5a at 10 nM pre-incubated with increasing doses of human monoclonal antibody HB8633 was added to HMC-1 cells prepared for calcium imaging recording. Immediately after recording the calcium flux, aliquots of anaphylatoxin–monoclonal immunoglobulin mixtures were subjected to the western blot assay. The magnitude of anaphylatoxin-induced calcium flux from HMC-1 cells decreased as the dose of monoclonal antibody increased, consistent with the data presented above (Fig. 1*d* and *e*). In addition, western blots showed a correlation between the magnitude of anaphylatoxin neutralization and the ratio of immunoglobulin-bound to free anaphylatoxins. At the lowest dose of monoclonal antibody, virtually all C5a was unbound and there was no inhibition of calcium signaling (Fig. 5*c*, lane 1); at the intermediate dose, the ratio of free to bound C5a was roughly 50:50 (Fig. 5*c*, lane 2) and there was a 47% inhibition of the calcium flux. At the highest concentration of monoclonal antibody, all C5a was bound to immunoglobulin (Fig. 5*c*, lane 3) and there was a complete abrogation of the calcium flux. Similar patterns of binding and functional anaphylatoxin inhibition, relative to the dose of monoclonal antibody, were obtained for C3a (data not shown).

We then modeled the C3a and Fab molecules for candidate sites that could be involved in binding. Three residues with shifted pK values may serve as binding sites for highly positively charged C3a molecules. Two of the residues were in the CH1 domain (tyrosine 305 and 358) and one was in the hinge region (lysine 255) of the Fab molecule (Fig. 5*b*). Electrostatics in the vicinity of the three residues showed a strong electric field oriented along grooves that are complementary in shape to C3a (Fig. 5*d*). We conducted a Monte Carlo sampling of 1.5 million possible binding modes and determined both the electrostatic and van der Waals energy components of the Fab-C3a interaction. Molecular dynamics simulations and cluster analysis of the lowest-energy conformations showed that the largest number of C3a molecules was centered on tyrosine 358 of the Fab fragment, indicating that this residue is

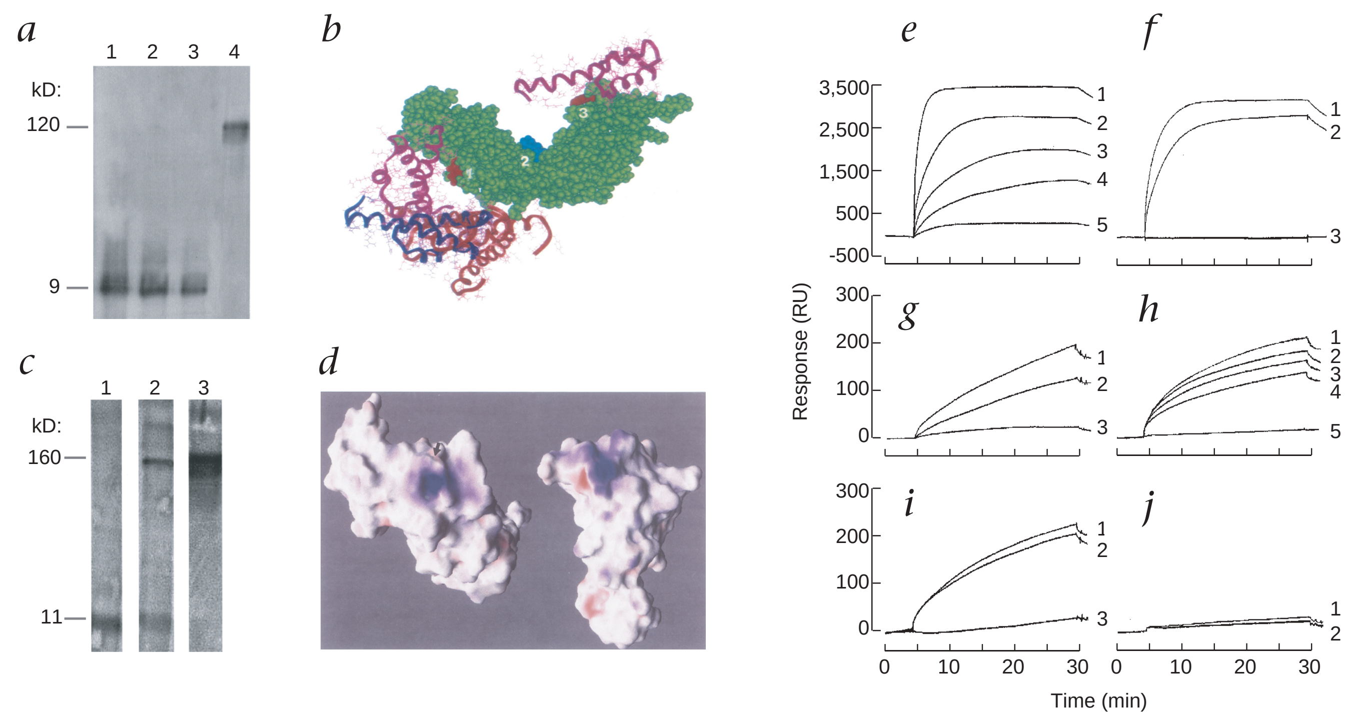

Fig. 5 Physical association of anaphylatoxin and immunoglobulin molecules. ***a***, Western blot analysis of C3a-F(ab)′$_2$ complex formation. Lanes were loaded with C3a alone (1), C3a pre-incubated with Fc IgG fragments (2), C3a pre-incubated with human serum albumin (3) and C3a pre-incubated with purified F(ab)′$_2$ (4). ***b***, Molecular modeling of potential binding sites for C3a in the Fab molecule and clustering of low-energy structures in their vicinity. Abnormally protonated residues in the Fab fragment are tyrosine 358 (1), lysine 255 (2) and tyrosine 305 (3). ***c***, Correlation between inhibition of C5a and C5a–monoclonal antibody complex formation. Lanes were loaded with a mixture of 10 nM C5a and 1 mg/ml (1), 12.5 mg/ml (2) or 25 mg/ml (3) monoclonal antibody HB8633, pre-incubated for 15 min. ***d***, Hypothetical orientation of both molecules before 'docking'. Arrow indicates position of tyrosine 358. The presence of grooves and clefts in its vicinity could allow for the N-terminal part of C3a to be accommodated after the initial electrostatic binding. ***e***, SPR sensorgram showing the binding of C3a-specific monoclonal antibody K13/16 to immobilized native C3a. The antibody concentration ranged from 3.9 nM (5) to 1,000 nM (1). ***f***, Binding of C5a-specific polyclonal antibody (1) and C5a-specific monoclonal antibody G25/2 (2) to immobilized C5a; binding of the monoclonal antibody to control peptide (3). ***g***, Sensorgram showing kinetics of F(ab)′2-IVIG binding to immobilized C3a (1), C5a (2) or blank sensor surface (3). ***h***, Sensorgram showing binding of 1,000 nM human monoclonal antibodies HB8501 (1), HB 8633 (2), HB 8636 (3) or 447-52D (4) to immobilized C3a, and interaction of HB8501 with control peptide (5). ***i***, Binding of HIV-specific antibody 447-52D to coupled C3a in the presence (1) or absence (2) of saturating amounts of the specific antigen, gp120; binding of 447-52D to the blank sensor chip (3). ***j***, Sensorgram showing injection of 1,000 nM Fc fragments of immunoglobulins over C3a (1)- and C5a (2)-coupled flow cells. All sensorgrams are representative of 3 identical experiments. RU, resonance units.

part of the binding site for C3a within the Fab fragment of the immunoglobulin molecule (Fig. 5*b*).

In order to better characterize F(ab)′$_2$-anaphylatoxin binding, we immobilized C3a and C5a on sensor chips and analyzed binding capacities and curves of different immunoglobulin preparations by SPR. C3a-specific (K13/16) and C5a-specific (G25/2) monoclonal antibodies had high binding capacities for their corresponding antigens, with rapid saturation (Fig. 5*e* and *f*). Similar binding characteristics were observed for a polyclonal C5a-specific antibody (Fig. 5*f*), a polyclonal C3a-specific antibody, and other monoclonal C3a-specific (K17/1) and C5a-specific (C17/5) antibodies (data not shown). In contrast, F(ab)′$_2$ -IVIG bound to C3a and C5a with 14- to 18-fold lower capacity than the anaphylatoxin-specific antibodies; its binding curve was linear and did not reach saturation, indicating complex binding kinetics (Fig. 5*g*). Identical results were obtained for F(ab)′$_2$ fragments purified from the F(ab)′$_2$-IVIG preparation (data not shown). We then tested human monoclonal antibodies with known specificity, one from each IgG subclass, for their capacity to bind C3a. We determined that their binding capacities and kinetics were similar to those of F(ab)′$_2$-IVIG (Fig. 5*h*). Furthermore, we saturated binding sites of the HIV-specific human monoclonal antibody with the recombinant gp120 antigen and showed that the binding capacity of this antibody for C3a was not affected by the presence of the specific antigen (Fig. 5*i*). The Fc fragment of the immunoglobulin molecule showed no binding to immobilized anaphylatoxins (Fig. 5*j*), in accordance with the results of our calcium studies. We observed no significant binding to control peptide or blank sensor surface for either of the analyzed immunoglobulins (Fig. 5*g*–*i*).

Discussion

The data provided here indicate that the biological activity of C3a and C5a anaphylatoxins, potent inducers and amplifiers of the inflammatory response, is attenuated by F(ab)′$_2$ fragments of IgG. Formation of anaphylatoxin-F(ab)′$_2$ complexes, as suggested by molecular modeling and confirmed by the shift in molecular weight of anaphylatoxins in the western blot analysis, interferes with binding of C3a and C5a to their receptors. This results in the inhibition of the anaphylatoxin-induced rise in cytosolic calcium in human mast cells as well as the release of TXB_2 and histamine from mast cells and whole-blood basophils. Proof of the *in vivo* ability of F(ab)′$_2$ molecules to interact with anaphylatoxins was obtained from studies of circulatory changes in pigs and from a mouse model of asthma. Pretreatment of pigs with F(ab)′$_2$-IVIG prevented the occurrence of C5a-induced anaphylactic shock (characterized by immediate circulatory collapse), even after repeated injections of lethal doses of C5a. IVIG treatment before and after the airway challenge in ovalbumin-sensitized mice led to a significant decrease in cellular migration into the bronchoalveolar and lung tissue compartments. Similar observations were made in mice homozygous for the *C3* gene deletion, supporting the hypothesis that immunoglobulin molecules exert their effect through binding and neutralizing anaphylatoxins. Previous studies[22,23] of animals with C3a receptor deficiency implicated C3a in asthma. Our findings support the role of C3a in the pathogenesis of cellular infiltration in asthma, and scavenging of anaphylatoxins as the possible mechanism of effect of IVIG. SPR analysis indicated that the non-variable region of Fab is involved in neutralizing anaphylatoxins. Human monoclonal antibodies of irrelevant specificity bound to anaphylatoxins, even in the presence of saturating amounts of the antigen. Furthermore, their binding characteristics and capacities were identical to those of Fab fragments and IVIG preparations, but distinct from those of anaphylatoxin-specific antibodies.

The specificity of the anaphylatoxin-neutralizing effect of immunoglobulin molecules was confirmed by showing that appropriate controls (human serum albumin, vehicle for immunoglobulin preparations and TGF-β2, a potential immunosuppressant) do not inhibit anaphylatoxins. We also excluded the specific anaphylatoxin inhibitors carboxypeptidase N and R from our IVIG preparations. In addition, highly purified human monoclonal antibodies of irrelevant specificity are as effective at inhibiting anaphylatoxins as are IVIG preparations. Although SPR data indicated that anaphylatoxin-specific antibodies do not have a role in the observed neutralization of C3a and C5a, their presence in pooled polyclonal immunoglobulin preparations cannot be ignored. If present, their beneficial effect in terms of anaphylatoxin neutralization could be viewed as additive to the inhibition through the constant region of the Fab fragment.

It has been suggested that IVIG exerts an anti-inflammatory effect by triggering Fc-γRIIB inhibitory receptors[24,25]. The mechanism that we propose in this study is distinct, as anaphylatoxin-neutralizing capacity was shown for both Fab and whole IVIG, but not for Fc fragments of IgG, in models and systems virtually free of IgG–Fc-γRIIB interactions. Whole immunoglobulin preparations (with both Fab and Fc fragments intact), however, could act simultaneously through both mechanisms, or predominantly one or the other (depending on a given pathological condition) to suppress deleterious inflammatory reactions.

Potentially harmful fragments of complement components are generated *in vivo* under physiologic conditions of low-level complement activation[26]. This process can potentially lead to cell damage mediated by C3b and autoimmune phenomena caused by C5a (resulting from its ability to activate endothelial cells and augment humoral immune responses[27–29]). However, harm to the host is controlled by regulatory complement proteins and possibly by immunoglobulins.

In spite of the high physiological concentration of IgG in serum, the ability of this endogenous pool to attenuate pathological conditions mediated by high levels of anaphylatoxins is restricted as a result of its rather inefficient binding of C3a and C5a. Slow and concentration-dependent uptake of C3a and C5a by immunoglobulins would allow preferential binding to target cells that express high-affinity receptors for these ligands[30]. In other words, at physiologic concentrations, IgG molecules seem to be at a disadvantage when it comes to competing for ligand binding with natural anaphylatoxin receptors. In addition, not all IgG clones may be capable of binding anaphylatoxins. Anaphylatoxin scavenging may be a function of a subset of IgG molecules, and would be a property not related to any current phenotypic markers used to classify immunoglobulins. Infusion of greater-than-physiological doses of exogenous immunoglobulins, in the form of IVIG, probably provides the critical mass of immunoglobulin molecules necessary to accelerate the uptake of anaphylatoxins by immunoglobulins or sufficiently increase the number of IgG molecules capable of binding anaphylatoxins.

In summary, our data indicate that the physical association of immunoglobulin molecules with C3a and C5a may result in neutralization of their biological effects *in vitro* and in relevant animal models of anaphylatoxin-mediated pathology. Such neutralization could represent a novel effector function of endogenous immunoglobulin molecules and also explain part of the anti-inflammatory effect of greater-than-physiologic doses of immunoglobulins modified for intravenous use, possibly extending their clinical applications.

Methods

Cell lines. HMC-1 cells[31] were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, non-essential amino acids, 50 U/ml penicillin and 50 mg/ml of streptomycin, at 37 °C in 5% CO_2. Human hybridoma cell lines HB8633, HB8501 and HB8636 were purchased from the American Type Culture Collection (Manassas, Virginia) and grown in serum and protein-free medium (Invitrogen, Carlsbad, California). Antibodies were purified by protein G column and their purity (>99%) was assessed by SDS-PAGE and densitometry.

Single-cell calcium imaging. $[Ca^{2+}]_i$ in HMC-1 cells was monitored using a single-cell imaging system (Atto Instruments, Rockville, Maryland). Cells (0.5×10^6 cells/well) were stimulated with 1–112 nM of C3a and C5a (Calbiochem, San Diego, California) alone or C3a and C5a pre-incubated for 15 min at 37 °C with immunoglobulin preparations or control reagents. Changes in cytoplasmic calcium, expressed as a ratio of 340 nm over 380 nm fluorescence, were recorded simultaneously in 23–76 cells.

Colorimetric assay for carboxypeptidases. The assay was performed as previously described[32].

Histamine release. Histamine assay was conducted using aliquots of HMC-1 cells (1×10^5 cells/ml) previously incubated with inhibitors or controls for 15 min at 37 °C, according to the manufacturer's instructions (Immunotech, Marseille, France). Total histamine was measured in cells subjected to lysis by 3 cycles of freeze-thawing. Percent histamine release was determined as the histamine measured in the supernatants divided by the sum of the histamine in the supernatants and cell pellet.

Whole blood histamine release assay. Heparinized whole blood samples from a healthy donor were stimulated with 56 nM C3a or 10 nM C5a alone, or the same concentrations of C3a and C5a were added to blood samples supplemented with 1, 5, 10 or 20 mg/ml IVIG. After 30 min at 37 °C, samples were centrifuged and histamine was measured in the cell-free supernatant using a specific ELISA kit (Immunotech). Total histamine content was measured from a 50-µl aliquot of white blood, diluted 1:20 in distilled water and subjected to 2 freeze thaw cycles.

Thromboxane release assay. HMC-1 cells at 1×10^5 cells/ml were incubated with anaphylatoxins alone, or with C3a and C5a pretreated with immunoglobulin preparations or controls. Thromboxane in the supernatants was measured using a commercial kit, following manufacturer's instructions (Amersham, Piscataway, New Jersey).

Animals. Balb/c mice were obtained from the National Cancer Institute (Frederick, Maryland). B6/129 F_1/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice deficient in *C3* (*C3*$^{-/-}$) were constructed as described[33]. Female Yorkshire swine (40–48 kg) were obtained from Archer Farms (Darlington, Maryland). All animal experiments were performed in compliance with the National Institutes of Health and Walter Reed Army Medical Research Institute guidelines.

Mouse model of asthma. Balb/c, *C3*$^{-/-}$, and B6/129 F_1/J (wild-type) mice were sensitized and challenged with ovalbumin. Bronchoalveolar fluid cell analysis[34] was done at 72 h. Quantitative histopathologic evaluation of inflammation severity was performed on entire H&E-stained lung sections by counting blood vessels cuffed by infiltration of eosinophils and mononuclear cells.

Closed-chest-instrumented pig. Anesthetized pigs were subjected to jugular vein and pulmonary and femoral artery cannulation for blood sampling, pressure recordings and volume control.

Western blot analysis. Electrophoretically separated proteins[35] were transferred onto Immobilon PVDF membranes (Millipore, Bedford, Massachusetts) that were then incubated with monoclonal antibodies against C3a and C5a and peroxidase-labeled mouse-specific goat IgG (Kirkegaard & Perry, Gaithersburg, Maryland). The antigen-antibody reaction was detected by use of enhanced chemiluminescence reagents (Amersham).

Molecular modeling. The possible binding modes of C3a and Fab were explored using a docking algorithm implemented with CHARMM[36]. A grid search using cylindrical coordinates oriented along the principal axes of Fab was conducted. Orientations of C3a were explored with rotations about its center of mass at each grid point. Grid spacing was 2 Å along Fab (R) and 30° around the center of mass of Fab (θ) and C3a (ϕ, ψ). Energetics were calculated with a distance-dependent dielectric to approximate solvent effects. Protonation states were determined using the Finite Difference Poisson-Boltzman method[37] implemented in MEAD and InsightII (Tripos Associates, St. Louis, Missouri). GRASP software (1.36) was used to analyze electrostatic maps.

Biosensor analysis. SPR measurements were taken using the BIACORE 3000 system (BIAcore, Uppsala, Sweden). Protein ligands (C3a, C5a and C1 peptide–endothelin receptor fragment[38]) were diluted to 10–20 µg/ml in running buffer and immobilized onto the carboxymethylated dextrane surface of a CM5 sensor chip (BIAcore) using the amino-coupling kit (BIAcore). Equivalent volumes of various immunoglobulin analytes at the same concentration (1,000 nM) were injected simultaneously through 4 flow cells over immobilized ligands.

Inhibition ELISA. Human-derived monoclonal antibody 447-52D against the V3 loop of HIV gp120 (0.13 µg/ml; NIH AIDS Reagent Program, Rockville, Maryland) was incubated overnight at room temperature with serial 3-fold dilutions of gp120, starting at 120 µg/ml. Bound and free antibody were transferred to a gp120-coated microtiter plate. Antibody binding was detected with alkaline phosphatase–labeled human-specific goat IgG (ICN/Cappel, Aurora, Ohio), followed by incubation with *p*-nitrophenyl phosphate (Sigma, St. Louis, Missouri) and optical density reading at 410 nm.

Acknowledgments
The authors thank T. Semere for his help with HMC-1 cell cultures; M. Opperman for monoclonal antibodies against C3a and C5a; P. Gronski for purified $F(ab)'_2$ immunoglobulin fragments and IVIG preparations (GammaVenin and Venimmun); R. Huber for the X-ray structures of the C3a molecule; E. Padlan for the X-ray and model structures of the Fab fragment of IgG; and B. Kelsall and P. Murphy for critical reading of the manuscript and helpful suggestions.

Competing interests statement
The authors declare that they have no competing financial interests.

RECEIVED 17 SEPTEMBER 2002; ACCEPTED 14 FEBRUARY 2003

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